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INDUCTION OF VESICLE FORMATION IN A CELL LINE DERIVED FROM IMAGINAL DISCS

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SUMMARY

The ability of insect hemolymph to induce vesicles in a high passage insect cell line, IAL-TND1, is described. The factor responsible, designated VPA for 'vesicle-promoting activity", was determined to be heat sensitive, nondialyzable, and protease Type XIV sensitive but insensitive to trypsin digestion. In efforts to determine the source of VPA, hemolymph was collected from different developmental stages of *Trichoplusia ni*, and certain tissues from *T. ni* were cocultured with IAL-TND1 cells. Hemolymph from every developmental stage tested exhibited VPA although the effect was somewhat reduced in spinning-stage larvae. Additionally, several tissues, including fat body, tess, and imaginal discs, released VPA into the culture medium. Neural tissues and endocrine glands did not induce vesicle formation.

Key words: insect cell line; *Trichoplusia ni*; hemolymph; vesicle-promoting activity.

INTRODUCTION

The mechanisms that control cellular morphology in vitro are of interest to cell culturists because of the possible relationships to developmental processes of tissues and organs in vivo. We previously reported the establishment of continuous cell lines that grew as histiotypic, multicellular vesicles (5,7) but which underwent a spontaneous morphological transformation after a year in culture to an aggregate (clumped) form. During pathology studies on these aggregate cultures, vesicles were formed after the addition of insect hemolymph to the cultures.

We now provide more detailed analyses of the response of aggregate cultures to the vesicle-promoting activity (VPA) of hemolymph and describe some early efforts to characterize the nature and possible source of the factor responsible for the change in morphology. The regulation of vesicle formation in our insect cell line is compared with that of vesicle and dome formation in mammalian cell lines (4,8,9).

MATERIALS AND METHODS

Cell cultures. The IAL-TND1 cell line developed from *Trichoplusia ni* imaginal discs by Lynn et al. (7) was used. The cells utilized in these studies were between the 60th and 210th subcultures (representing a minimum of 160 cell doublings since the line was initiated). The cultures consisted predominantly of aggregates and single cells (few or no vesicles) since the 40th subculture. Cells were grown in Grace's (3) media (GIBCO, Grand Island, NY) as modified by Yunker et al. (11) with 20% (vol/vol) heat-treated fetal bovine serum (FBS) (Gainesville laboratory) or in a 3:1 ratio of Goodwin's IPL-52B and IPL-76 media (2) (K. C. Biological, Lenexa, KS) supplemented with 9% (vol/vol) FBS (Beltsville laboratory). Cells were maintained at 28° C and subcultured weekly as described previously (7).

Assay procedure. Vesicle promoting activity was measured by distributing IAL-TND1 cells and aggregates in multiwell plates (4, 24, or 96-well plates from Nunc, Roskilde, Denmark; Costar, Cambridge, MA; or Corning, Corning, NY) in the test media and counting total numbers of vesicles per well identified by their distinctive morphology through an inverted microscope (Leitz or Wild). Inasmuch as the integrity of the aggregates at lower passages was too great for isolating single cells for counting, the cell numbers varied in different experiments. In general, 1×10^4 cells/well were used in 96-well plates with proportionally greater numbers in the larger well plates relative to the surface area. Typically, vesicles were counted after 2 and 6 d of exposure to the test media. The variation in the background (control) number of vesicles in these studies was a function of passage level and media used in the various experiments. Thus, numerical comparisons can not be made between different experiments.

Hemolymph treatments. Trichoplusia ni larvae were bled by cutting a proleg and collecting the hemolymph into cold test tubes that contained culture medium supplemented with 0.03% cysteine to reduce melanization. The concentration of hemolymph was determined

by the difference in volume before and after the insects were bled. The medium containing hemolymph was centrifuged (100 ×g, 5 min) and sterilized by filtration (0.22 μm). This procedure removed contaminating whole hemocytes and fat body cells. Although the possibility exists that some of these cells could have lysed, we do not believe that lysis accounts for very much of the material in the test media. Hemolymph was also collected from

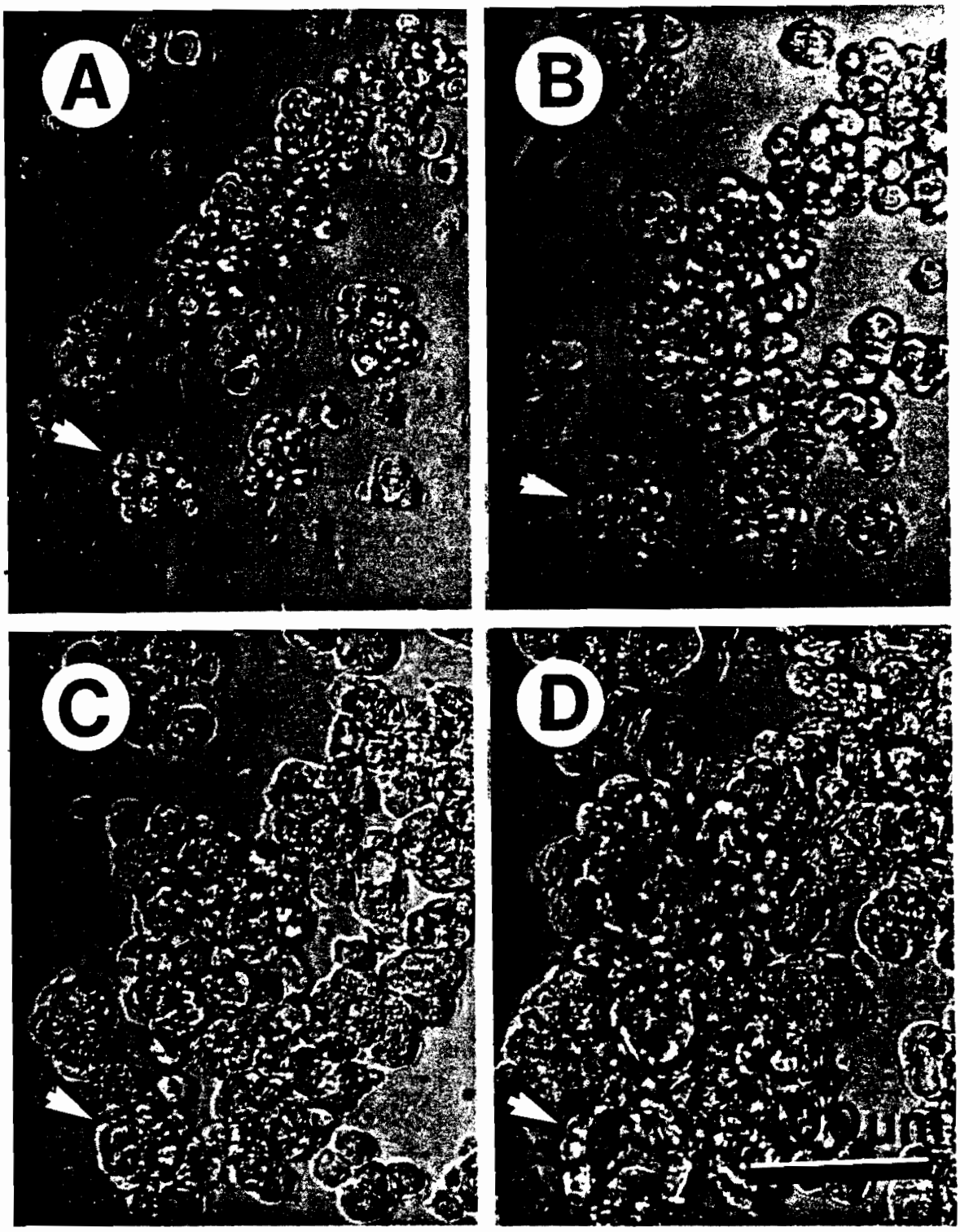

FIG. 1. Photomicrographs of IAL-TND1 during a typical experiment with exposure to 2% *T. ni* hemolymph. *A*, at time of hemolymph addition; *B*, 24 h exposure; *C*, 48 h exposure; *D*, 4 d exposure. The *arrow* in each frame indicates the same aggregate of cells over the 4-d period showing the formation of a vesicle in response to the hemolymph. *Marker bar* = 100 μm.

penultimate (IVth) instar larvae, early last (Vth) instar larvae, late Vth instar, spinning Vth instar larvae, and pupae to examine the effects of donor developmental stage.

Heat treatments. Fifty microliters hemolymph from Vth instar *T. ni* was treated in 20, 60, and 100° C (boiling) water baths for 5 or 30 min, then diluted to a final concentration of 2% in culture medium that contained 0.03% cysteine for testing on IAL-TND1 cultures as described above.

Dialysis. Hemolymph collected from Vth instar larvae of *T. ni* was concentrated and dialyzed simultaneously with a ProDiMem apparatus using 10 000 and 60 000 mol wt cut-off membranes. The membranes were boiled in a solution of 2% sodium bicarbonate + 1 m*M* EDTA, rinsed in distilled water, then boiled in distilled water for 10 min. Hemolymph (0.4 ml) was diluted with 9.6 ml of Grace's medium (20% FBS, 0.03% cysteine), Grace-Yunker's medium, or Ringer's solution. The diluted samples were filtered (Nalge nylon filter) and 1.0 to 2.5 ml was dialyzed and concentrated to approximately 0.5 ml at 4° C for 24 h. The concentrates were diluted to 2.5 ml with the appropriate solution, filtered, and 10 to 40 μl was assayed.

In one experiment, equilibrium dialysis was carried out with a six-cell plexiglass apparatus (MRA, Boston, MA) and 60 000 dalton cut-off membrane (ProDiMem). One and a half milliliters of 4% hemolymph (diluted with Grace's medium containing cysteine and 20% FBS) was placed on one side of the membrane and 1.5 ml of Grace's medium only on the other side in each cell. The unit was placed on a shaker at 4° C for 24 h and 10 μl aliquots were assayed.

Protease sensitivity. Hemolymph was collected into Hanks' buffered saline without Ca^{++} or Mg^{++} and with EDTA and 0.03% cysteine. The initial solution was 10 to 20% hemolymph, which was centrifuged (500 ×g) and filtered (0.2 μm). Trypsin (BBL, Cockeysville, MD) was added at 0.25% to the hemolymph solutions and incubated at 28° C for 30 min. (This temperature was used instead of the optimal 37° C to prevent possible inactivation of the VPA.) After incubation, the hemolymph solution was mixed with culture media that contained FBS and 0.6 mg soybean trypsin inhibitor (7750 U/mg, Schwartz/Mann, Orangeburg, NY) per milliliter to a final concentration of 1% hemolymph. The positive controls were treated similarly (in saline with EDTA and cysteine and diluted with medium containing FBS and inhibitor) but without trypsin. A second experiment was performed on hemolymph, which had been fractionated by centrifugation (5000 ×g, 30 min, 4° C) of 10% hemolymph in saline through a Centricon-30 ultrafiltration device (Amicon Corp., Danver, MA) with two saline rinses of the retained fraction. The volume of the retained fraction was adjusted back to the original volume with saline before trypsinization. Trypsin activity was measured in the retained hemolymph fraction by addition of 1 m*M* α-*p*-Tosyl-L-arginine methyl ester (TAME, Sigma Chemical Co., St. Louis, MO) and measuring absorbancy at 247 nm with time. A third experiment was performed with protease Type XIV (Sigma) instead of trypsin. The retained hemolymph fraction (10%) was treated 30 min at 28° C with 1 mg enzyme (6 U/mg) /ml and subsequently diluted to 1% hemolymph with culture medium containing FBS and trypsin inhibitor as above. Hemolymph-free controls contained equivalent concentrations of enzyme, media, and inhibitor whereas the positive control contained retained hemolymph fraction treated at 28° C for 30 min without enzyme before dilution with media containing FBS and inhibitor.

Co-culture with T. ni tissues. The IVth or Vth instar larvae were surface sterilized in 70% ethanol for 10 min, rinsed twice with sterile distilled water, and dissected while in culture medium. Tissues were rinsed three times in medium and incubated at least 3 d before being transferred to multiwell plates with IAL-TND1 cells. The rinses and preincubation time insured that a minimum of hemolymph was transferred with the tissues and care was taken to ensure a minimum of contamination of the test tissues by other tissues from the larvae. In a second test, the tissues had been in culture 2 wk before testing with IAL-TND1 cells. Tissue-conditioned media on the tissue cultures for the week before the test were collected and filtered (0.2 μm) before assaying on IAL-TND1 cells.

Results and Discussion

The effect of hemolymph exposure on the formation of vesicles in IAL-TND1 cultures can be seen from a typical

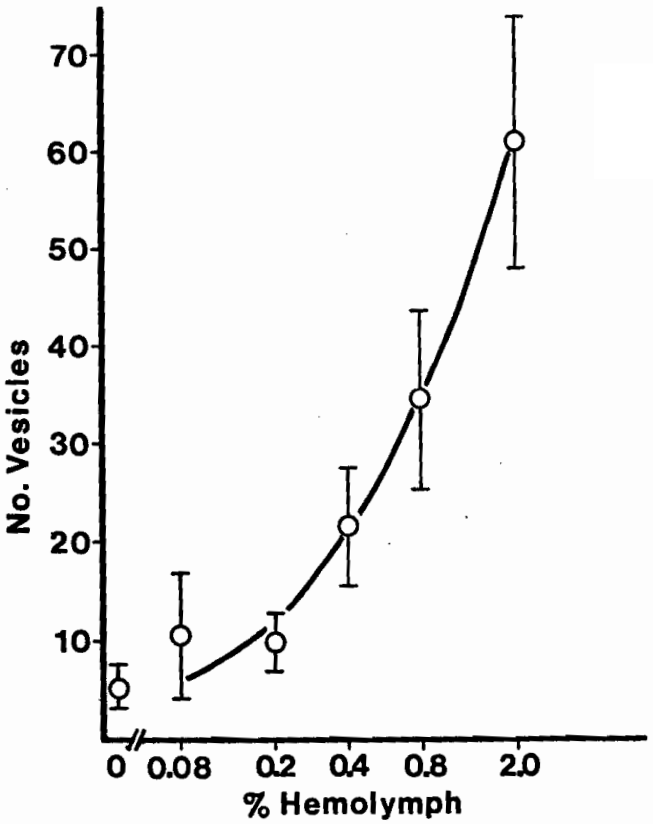

FIG. 2. Effect of *T. ni* hemolymph concentration on vesicle formation in IAL-TND1 cultures. Counts represent the mean number of vesicles per well of a 16-well plate after 3 d exposure to hemolymph (four wells/treatment).

TABLE 1

FACTORS INFLUENCING VESICLE PROMOTING ACTIVITY OF HEMOLYMPH[a]

Heat Treatment of Hemolymph[b]	Number of Vesicles/Well
Control (2% hemolymph)	34.0 ± 7.1
20° C, 5 min	46.0 ± 11.3
20° C, 30 min	51.5 ± 9.2
60° C, 5 min	0
Hemolymph-free control	0
Dialysis (tests on retained material)	>60 000 Dalton
2% Hemolymph, no dialysis	328 ± 31
2% Hemolymph in Yunker's dialyzed[c]	290 ± 14
Yunker's, no hemolymph, dialyzed	24 ± 14

[a]This represents 6-d counts on all vesicles/well.

[b]Heat treatments were made on hemolymph before mixing with the culture media. In addition to 60° C, 5 min., hemolymph was also treated at 60° C for 30 min, and 100° C for 5 and 30 min with no VPA remaining post-treatment. Tests were performed in Nunc 4-well plates with two wells per treatment. Data represent mean ± standard deviation.

[c]Tests of hemolymph diluted in Grace's medium or Ringer's solution yielded similar results, as did dialysis with a lower (10 000 dalton) pore-size membrane. Tests were performed in Nunc 4-well plates with two wells per treatment. Data represent mean ± standard deviation.

experiment (Fig. 1). The vesicles form from existing aggregates of cells and not from single cells or as a result of new aggregations. Figure 2 shows a dose-response curve similar to the effects we reported previously (6) and a concentration-dependent effect at 3 d. However, when this experiment was continued for longer exposures (8 d) a maximal response (>100 vesicles/well) was observed at every level of hemolymph tested, which indicated that VPA was needed at only low levels when present in the cultures for sufficient time. An incubation of 1 wk in medium that contained 2% hemolymph resulted in vesicle formation during 9 successive weekly subcultures without further hemolymph treatment. The cultures formed clumps in the 11th and 12th wk of this experiment.

The effect of various treatments on the ability of hemolymph to induce vesicle formation is shown in Tables 1 and 2. The heat treatments indicate the factor is heat labile because it was completely inhibited by 5 min exposure to 60° C. The molecular weight was shown to be greater than 60 000 by dialysis. Equilibrium dialysis showed no activity in the distal compartment, which indicated that no VPA was present in the lower molecular weight fraction. The knowledge of the relatively large size of VPA made the effect of trypsin exposure somewhat unexpected (Table 2). In an early test, whole hemolymph was diluted with saline and treated with trypsin without reducing VPA.

Ultrafiltration was used to purify VPA partially in case other components in the hemolymph interfered with the enzyme activity. The trypsin still did not affect VPA levels even though the TAME assay proved the trypsin was active. One possible explanation for these results might be that the VPA contains low arginine and lysine quantities thereby imparting low trypsin sensitivity. Subsequently, a test was run with protease XIV, a nonspecific protease from *Streptomyces griseus*. This enzyme was effective in reducing VPA (Table 2).

In another series of experiments, we investigated the source of the VPA (Table 3). The factor was present in hemolymph from every developmental stage of *T. ni* tested, although somewhat reduced in spinning-stage Vth instar larvae (Table 3 *A*). Thus, VPA was not closely tied to developmental stages and therefore is probably not dependent on changes in titers of developmental hormones such as juvenile hormone and ecdysone. Additional evidence of this was supplied by coculturing IAL-TND1 cells with various larval tissues (Table 3 *B*). In these experiments, several different tissues promoted vesicle formation. Preliminary tests showed VPA from cultured fat body, silk glands, testes, and imaginal discs, although no effect was seen from various nerve tissues (brain, subesophageal ganglia, thoracic ganglia) or from endocrine glands (corpora cardiaca-allata complex or prothoracic glands) (Test 1, Table 3 *B*). Subsequent experiments with some of these tissues as well as cell-free media collected from *T. ni* fat body and imaginal disc cultures showed similar results and indicate the VPA was released into the culture media. Comparison of VPA levels from different tissues is difficult because the amount of tissue used in the different tests varied. It was interesting, however, to note the high VPA from imaginal discs, the homologous tissue to the cell line, which could indicate that some tissue specificity for VPA existed.

The diverse range of tissues capable of producing VPA is an indication that it is not a hormone. Although we do not have evidence that the factor is a mitogen in the sense that it promotes cell division, we believe it may share some properties with the peptide growth factors found in vertebrates, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), or the somatomedins [*See* (10) for review]. Such factors may have played a role

TABLE 2

EFFECT OF ENZYME TREATMENT ON HEMOLYMPH VESICLE-PROMOTING ACTIVITY

Protease Treatment[a]	Total Hemolymph Trypsin	High Molecular Weight Fraction[b] Trypsin	High Molecular Weight Fraction[b] Type XIV
1% Hemolymph, no enzyme	79.3 ± 32.2[d]	140.7 ± 35.5[d]	63.5 ± 17.1[f]
1% Hemolymph, enzyme[c]	71.5 ± 22.4[d]	135.2 ± 29.3[d]	2.7 ± 0.8[e]
Hemolymph-free control	10.3 ± 4.4[e]	35.7 ± 11.0[e]	4.5 ± 1.4[e]

[a]Enzyme treatments were performed on concentrated hemolymph (10 to 20% equivalent) for 30 min at 28° C. The hemolymph was then diluted to 1% equivalent for testing on cells. Data represent mean total number of vesicles ± standard deviation per well in 96-well Corning culture plates (six wells/treatment). Numbers within each column followed by the same letter are not significantly different ($\alpha > 0.025$) (Kruskal-Wallis).

[b]Fractionated hemolymph was prepared from the material retained by a Centicon-30 and rediluted to the original volume.

[c]Trypsin was at 0.25% during hydrolysis and protease Type XIV was used at 1 mg/ml.

TABLE 3

SOURCE OF VESICLE PROMOTING FACTOR

	Number of Vesicles per Well	
A. Hemolymph Donor Age[a]	2 Days	6 Days
Penultimate, instar larvae (73 mg)	19.0 ± 9.0**	>100
Early last, instar larvae (93.4 mg)	22.5 ± 3.0**	>100
Late last, instar larvae (295 mg)	17.0 ± 3.8**	>100
Spinning (188 mg)	8.75 ± 3.5	62.0 ± 4.25
Pupae (171 mg)	33.75 ± 10.3**	>100
Hemolymph-free control	4.25 ± 1.0	14.5 ± 4.5
B. Tissue Coculture[b]	Test 1	Test 2
Hemolymph (1.5%)	57.8 ± 5.4*	-
Brain	31.7 ± 5.8	-
Subesophageal ganglia	37.7 ± 6.4	-
Thoracic ganglia	33.2 ± 8.3	43.2 ± 8.3
CFCM-thoracic ganglia[c]	-	30.0 ± 8.7
Corpora cardiaca/allata	33.25 ± 9.4	61.0 ± 14.7
CFCM-corpora cardiaca/allata	-	32.2 ± 8.4
Prothoracic gland	31.0 ± 14.0	-
Silk glands	40.0 ± 22.5	-
Fat body	55.5 ± 13.4*	76.0 ± 7.7*
CFCM-fat body	-	156.8 ± 25.2**
Testes	48.25 ± 17.1*	-
Imaginal disc	142.7 ± 8.6**	161.8 ± 38.3**
CFCM-imaginal disc	-	225.3 ± 73.9**
Hemolymph-free control	30.3 ± 11.1	36.3 ± 10.2

[a]Data represent the mean number of vesicles ± standard deviation per well in four-well Nunc culture plates resulting from exposure to 2% hemolymph from each source. Treatment effects were compared to the control in each test with Miller's large sample approximation of a distribution-free multiple comparison test based on Kruskal-Wallis rank sums. ** Indicates level of significance, $\alpha > 0.05$. Sample size, $n = 4$ per treatment.

[b]Data are as in *A*, except tests were run in 96-well Corning culture plates and counts were made after 6 d exposure to tissues or media. Sample size per treatment, $n=4$ in Test 1 and $n=6$ in Test 2. * Indicates level of significance, $\alpha > 0.10$; ** significance, $\alpha > 0.05$. Differences in hemolymph-free control vesicle numbers between *A* and *B* in this table were probably due to different passage levels (68 versus 210, respectively), media, and numbers of cells used in the different tests.

[c]CFCM is cell-free conditioned media collected from tissue cultures of the respective *T. ni* tissues.

in maintaining vesicles in IAL-TND1 at low passage levels because high concentrations of serum were necessary to maintain vesicles (6). However, tests with EGF and FGF (Collaborative Research, Inc., Lexington, MA) at concentrations from 5 to 200 ng/ml and serum substitutes containing these factors (NuSerum, Collarative Research, Inc. and UltraSer G, LKB Instruments, Gaithersburg, MD) on high pasage IAL-TND1 cells did not induce vesicle formation, indicating that VPA from insect hemolymph differs from these vertebrate growth factors.

The possible relationship of the regulation of vesicle formation in our insect cell line to that of mammalian cell lines was considered. Dome formation in oral epithelia is prevented by ouabain and stimulated by dibutyryl cyclic AMP (1). Dome formation is also stimulated by dibutyryl cyclic AMP in rat and dog epithelial cell cultures (4) and in a lung adenocarcinoma cell line (9). Mauchamp et al. (8) found that hog thyroid cells in primary culture produce three-dimensional structures including vesicles and that prostaglandins and dibutyryl cyclic AMP stimulate follicle formation. In our studies, the simple puncture of an insect cellular vesicle with a microneedle caused the collapse of the vesicle. However, ouabain (Sigma) added to the culture medium in final concentrations of 10^{-6}, 10^{-8}, and 10^{-10} *M*, did not prevent either the formation or maintenance of vesicles. This is somewhat perplexing but probably does not exclude the possibility of active transport in this system. Additionally, the presence of 10^{-3} to 10^{-6} *M* dibutyryl cyclic AMP (Sigma) had no effect on vesicle formation; nor did 10^{-5} to 10^{-8} *M* prostaglandins E-1 and E-2 (Sigma). Therefore, it seems that the regulation of vesicle formation in insect cell lines is different from the regulation of dome and sphere formation in mammalian cell cultures. Manifestly, this cell line from insect imaginal discs provides a unique system for examining the regulation of cellular morphogenesis in vitro.

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